2-A-124

The Measurement of Phenol in Urine by Gas Chromatography as a Check on Benzene Exposure

A. B. VAN HAAFTEN, M.D., and S. T. SIE, Ph.D.

Koninklijke/Shell-Laboratorium, Amsterdam, Netherlands
(Shell Research N. V.)

Several methods for the determination of phenol in urine as a check on benzene exposure are compared. Known colorimetric methods have been tested against a gas chromatographic procedure. From the results it is concluded that the gas chromatographic procedure is to be preferred for routine testing.

Introduction

THE PUBLICATIONS of Teisinger and Fiserova-Bergerova[1] and of Walkley *et al.*[2] on the measurement of phenol in urine as an index of benzene exposure, or poisoning, have aroused much interest. They show that a linear relationship may be assumed between benzene exposure and the increase in the amount of phenol—which also occurs normally—in urine.

The phenol level in urine proves to be a more reliable index of benzene exposure than any of the following:

1. The ratio between inorganic and organic sulfates in urine, a criterion that is commonly used.
2. Periodic blood tests, because experience has shown that they fail to reveal chronic benzene poisoning at a stage when the damage done to the blood-producing organs is still reversible.
3. Occasional benzene determinations in the atmosphere, since they do not provide an accurate picture of the actual exposure of the worker throughout a working day.

The detection of excessive benzene exposure by the determination of phenol in the urine passed at the end of a working day is therefore an important advance in the toxicology of benzene.

We have compared various methods of carrying out this determination.

Phenol Determinations in Urine

The two most common methods for determining phenol are based on colorimetric measurements in the steam distillates of urine, namely: (1) Gibbs'[3] method, with the color reagent 2,6-dichloroquinone chlorimide; and (2) the method of Theis and Benedict,[4] with diazotized *p*-nitroaniline as the color reagent.

The first method is the more specific for phenol and produces lower values than the second. It is, however, more critical and takes much more time. For these reasons Walkley *et al.* preferred the method of Theis and Benedict, particularly for routine tests.

After an investigation in our laboratory had shown that phenol determination in urine by a gas chromatographic procedure (GLC) is quite feasible, we compared this method with the two methods already mentioned. The gas chromatographic method is described in the Details of Analysis.

The urine produced at the end of a working day by three workers was tested for phenol. It was known with certainty (by GLC analysis of air samples) that these persons had been exposed to a low concentration of benzene. The phenol contents, in milligrams per liter, were:

Theis and Benedict	Gibbs	GLC
66	44	30
36	15	12
60	30	31

The obvious question is: "Why do the three methods produce such widely divergent results?"

In discussing the differences between the two colorimetric methods, Teisinger[1] and

Walkley[2] do not give a satisfactory explanation. Teisinger did find that the differences probably are due to the variations in normal "phenol" content in urine. In persons exposed to benzene, subtraction of the "phenol" found by the respective methods in normal urine (before benzene exposure) gave about the same "extra" phenol values.

Our investigations revealed that both colorimetric methods suffer from interference by other phenolic compounds present in urine. In Theis and Benedict's method, *p*-cresol is codetermined; in Gibb's method, it is scarcely so. In both methods *m*- and *o*-cresol interfere. The GLC method is specific for the determination of phenol and also allows the separate determination of (*p*+*m*)-cresol and *o*-cresol.

These facts explain the differences in phenol content as determined by the three methods. When one considers that *p*-cresol is not a metabolite of benzene, Teisinger's conclusion on comparing the two colorimetric methods is now also clear. The varying values, especially of the normal *p*-cresol secretion in urine, will accordingly have an adverse effect on the reliability of the test, especially of the Theis and Benedict method.

With regard to the cresols, it is known[5] that *p*-cresol is normally present in urine, even in much larger quantities than phenol. Schmidt[6] found cresol secretions of 65 to 117 mg/per 24 hours (average 87 mg). Siegfried and Zimmerman[7] found 58% *p*-cresol as referred to the total phenols, the remainder being mainly phenol; other phenolic compounds were found only in minute quantities.

Phenol and *p*-Cresol in Normal Urin

Phenol Content

Much research work has already been done to ascertain the normal phenol content of urine. This content depends on several factors besides that of normal food intake—for instance, the use of medicines, spices, and condiments.

According to the test method applied, the average normal phenol content is variously reported:

Authors	Content (mg/liter)	Method
Porteous and Williams[8]	5–10 (uncorrected)	Gibbs
Teisinger and Fiserova-Bergerova[1]	9.5 (uncorrected)	Gibbs
Walkley and co-workers[2]	30 (corrected)	Theis and Benedict
Deichmann and Schäfer[9]	25 (uncorrected)	Theis and Benedict

We used the GLC method to ascertain the phenol content of the urine of a number of adults (male and female) who had not been in contact with benzene and who stated that they had not recently taken any medicine. The group average was 5 mg/liter (20 persons, 40 samples).

When the urinary phenol values were corrected for a standard specific gravity of 1.024, as recommended by Walkley and co-workers, a group average of 7.5 mg/liter was found. (See the Addendum.)

Figure 1 is a plot of the differences between the normal phenol concentrations in the urine of the test persons.

No significant difference in the group average was found between morning and afternoon urines with regard to the corrected phenol contents (morning urines, 8 mg/liter; afternoon urines, 7 mg/liter).

For persons smoking up to twenty cigarettes a day, no significant differences in urinary

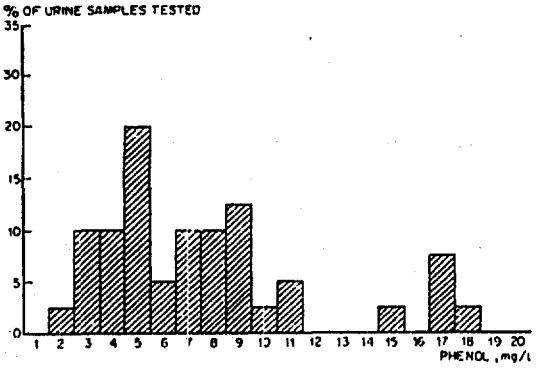

FIGURE 1. Histogram of normal phenol concentrations in urine of different persons (corrected values).

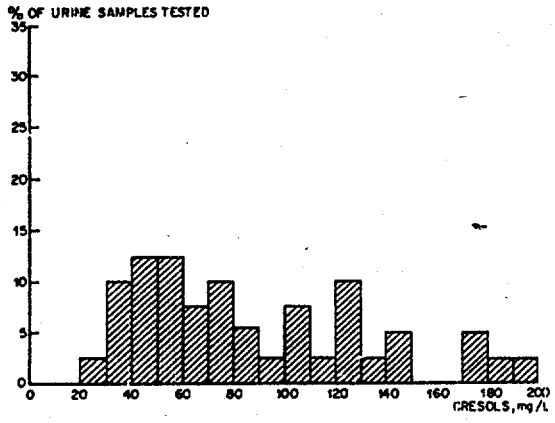

FIGURE 2. Histogram of $(p+m)$-cresol concentrations in urine of different persons (corrected values).

phenol content were found as compared with that in nonsmokers.

$(p+m)$-Cresol Content

In the same group of persons, the $(p+m)$-cresol content was also determined by the GLC method; the results are plotted in Figure 2. The group average was 89 mg/liter (corrected for density of urine).

Phenol in Urine of Persons Exposed to Benzene

Samples of morning urine (on rising) and of afternoon urine (4:30 PM) of workers slightly exposed to benzene during working days were examined by Theis and Benedict's method and by GLC, with the results shown in Table I.

TABLE I

Worker	Phenol Content (mg/liter)	
	Theis	GLC
A (morning)	59	18
(afternoon)	56	17
B (morning)	52	21
(afternoon)	57	21

Comparison of the morning and afternoon urines was repeated later by the GLC method, a third person being included who worked at a distance of some meters from the focal point of the benzene exposure. These results are shown in Table II.

It is evident from these values that no significant difference was found, by any of the methods applied, between morning and afternoon urines of the persons exposed to benzene. This does not agree with Walkley's findings.[2]

TABLE II

Worker	Phenol Content (mg/liter)
	GLC
A (morning)	30
(afternoon)	29
B (morning)	31
(afternoon)	29
C (morning)	12
(afternoon)	14

Details of Analysis

Principle of the Method

Urine samples are heated in the presence of phosphoric acid in order to hydrolyze the conjugated phenols (phenols bound as sulfates or glucuronates); the hydrolysis is carried out in a heated section upstream of the analysis column.

The liberated phenols are separated in a polyethylene glycol column and determined by means of a flame ionization detector. The method allows determination of phenol and cresols separately in urine in the concentration of 1 to 1000 mg/liter.

Apparatus

A flow diagram of the apparatus is shown in Figure 3. Most of the components are commercially available. The air thermostat containing the injection port, switching valve, columns, and detector is suitable for working temperatures up to 250°C and has an accuracy of approximately 0.5°C. The double four-way cock has a stainless-steel housing and a plug lined with reinforced Teflon and is suitable for temperatures up to approximately 200°C. The precolumns (hydrolysis sections) consist of 10-cm lengths of stainless-steel tubing (internal diameter 6 mm) filled with 30/50 mesh borosilicate glass powder. The analysis columns consist of 100-cm lengths of stainless-steel tubing (internal diameter 3 mm) filled with 60/80 mesh Chromosorb-W coated with 10% (w) polyethylene glycol 6000.

All metal parts that come into contact with sample components are made of stainless steel

and are kept at thermostat temperature (150°C).

The flame ionization detector has a nickel-tipped jet which acts as one of the electrodes, the other electrode being a cylinder of platinum foil surrounding the flame. A potential of 90 volts is kept between the electrodes; the current is measured across a resistor variable stepwise between 10^8 and 10^{10} ohms. The signal is measured by means of an impedance converter of sufficiently fast response and registered on a 2.5-mv full-scale potentiometric recorder.

Hydrogen used as the carrier gas (6 liters/hr) is purified by passing it through a column of 5-A molecular sieves at high pressure. Combustion air (100 liters/hr) and carbon dioxide (40 liters/hr) are purified by passing them over copper oxide at 800° to 900°C. The addition of carbon dioxide to the combustion air has been found to improve the signal-to-noise ratio of the detector. The background signal for a properly stripped column has been found to be sufficiently low to permit sensitive measurement (approximately 5×10^{-11} A at the conditions mentioned).

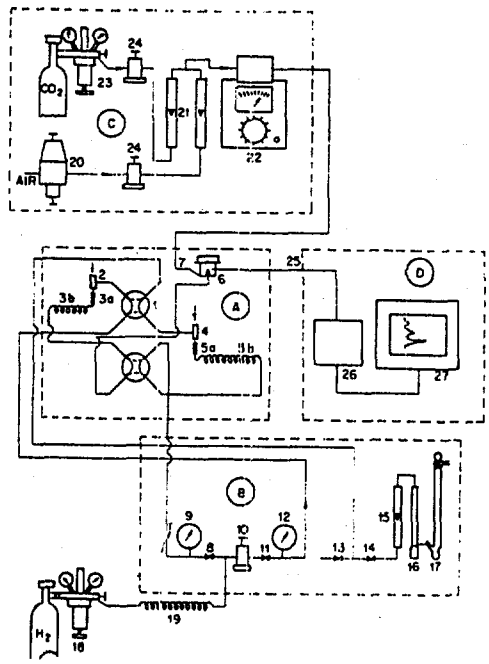

FIGURE 3. Flow diagram for analysis with flame ionization detection. *A, gas chromatographic circuit* (thermostatted): (1) double four-way cock; (2, 4) sample injection ports; (3a, 5a) precolumns (hydrolysis sections); (3b, 5b) analysis columns; (6) flame ionization detectors; (7) connection for combustion air and carbon dioxide. *B, carrier gas circuit:* (8) adjusting valve for purging gas; (9) manometer for leak testing; (10) precision reducing valve; (11) adjusting valve for carrier gas; (12) manometer for measuring column inlet pressure; (13) needle valve, connection used when measuring carrier gas flow; (14) needle valve for leak testing; (15) rotameter; (16) humidifier; (17) soap film meter; (18) reducing valve for hydrogen; (19) purifying column (molecular sieves, 5 A). *C, combustion air circuit:* (20) combined air filter–reducing valve; (21) rotameters; (22) furnace; (23) reducing valve for carbon dioxide; (24) precision reducing valves. *D, electrical part:* (25) nonmicrophonic shielded cable; (26) flame ionization detector amplifier; (27) potentiometric recorder.

Experimental Procedure

Urine samples that could not be analyzed immediately were stored in a frozen condition at –20°C in small polyethylene screw-cap bottles. Before the analysis, each urine sample was mixed with an equal volume of concentrated phosphoric acid (analytical grade, specific gravity 1.75). From this acid mixture a small amount (10 μl) was injected, by means of a precision microsyringe, into the hydrolysis column via the injection port.

Calibration was carried out in exactly the same way, with freshly prepared dilute solutions of known phenol and cresol contents instead of urine samples. The chromatograms were evaluated on a peak-height basis.

The slow elution of small amounts of heavy components from urine samples was found to cause a slight increase in the background signal after each injection. This did not interfere seriously, as the blank signal could be compensated electrically. When, however, the background signal became too high after a large series of injections, the double four-way cock was switched. The subsequent analyses were carried out with the spare column combination, while the used one was simultaneously stripped clean with a countercurrent stream of carrier gas. This arrangement is especially suitable for analyzing very large series of samples. When the number of samples is limited (to, say, less than 20 a day), a simple setup could be used, consisting of one column combination only without switching valve. Cleaning of contaminated columns may be carried out by maintaining the carrier gas flow overnight.

Results

Examples of chromatograms are given in Figure 4. As will be seen, phenol is well separated from cresols. Meta- and para-cresol

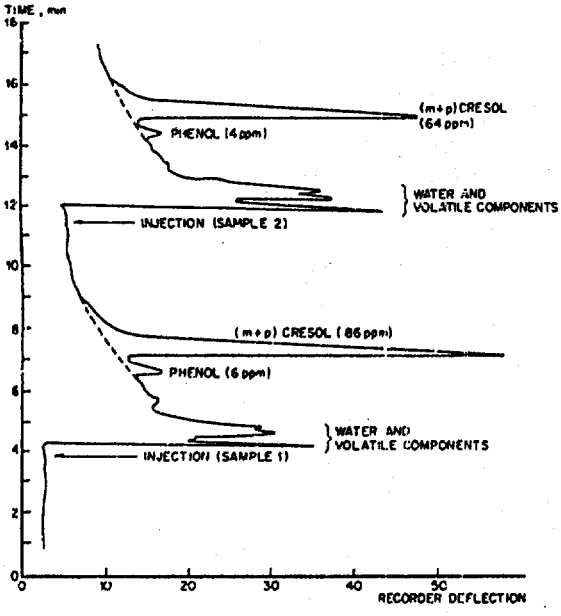

FIGURE 4. Examples of chromatograms. Sample size, 10 μl of urine-phosphoric acid mixture (1:1). Conditions of analysis: temperature, 150°C; carrier gas flow, 6 liters of H_2 per hour; column, 100 cm × 3 mm polyethylene glycol 6000 on Chromosorb-W; precolumn, 10 cm × 6 mm glass powder; measuring range, 100 mv at 10^9 ohms.

come together as one peak. Ortho-Cresol was found to be absent in the urine samples investigated.

The relationship between peak height and concentration is essentially linear between 1 and 1000 ppm (w/w) phenol or cresol. Good agreement was found between the phenol content experimentally found in urine samples to which known amounts of phenol had been added and the calculated values. The hydrolysis of conjugated phenols proved to be sufficiently quantitative for practical purposes.

The repeatability of the method was tested by carrying out a number of analyses on a urine sample containing approximately 30 ppm (w/w) of phenol; a standard deviation of 1.2 mg/liter (approximately 4% relative) was found. The sensitivity of the gas chromatographic method permits the detection of less than one ppm (w/w) of phenol in .5 μl of urine.

The analysis time compares very favorably with the time required for colorimetric methods; the gas chromatographic analysis can be carried out in less than 10 minutes.

Detection of Benzene Exposure

If the phenol content of urine is to be accepted as an index of benzene exposure, it will have to correlate as closely as possible with benzene concentrations in the atmosphere. It is only then that justified conclusions can be drawn concerning maximum permissible phenol concentrations in urine. That there are differences of opinion on these concentrations is apparent from the following:

Teisinger and Fiserova-Bergerova[1] conclude from their observations that a group average of 100 mg of phenol per liter in 24 hours' urine indicates that the worker has been exposed to a benzene atmosphere of 35 ppm.

Walkley and co-workers[2] conclude from their studies that a group average of 200 mg of phenol per liter of urine indicates exposure to 25 ppm of benzene.

If a few corrections are applied to make the conclusions of the two investigators comparable, the allowable phenol content of urine is still significantly lower in Teisinger's findings than in Walkley's.

The correlation between benzene concentration and "phenol" content in urine was plotted by Walkley as shown in Figure 5 (line 2). The slope of the line (angle λ) is the criterion here. Its correctness is determined by several factors, including the number of test persons and the reliability of the phenol determination. Walkley supposes the group average (69 persons) of the normal "phenol" content of urine to be 30 mg/liter. On exposure to 25 ppm the group average of "phenol" in urine will then be about 210 mg/liter.

Conversely, from a group average of about 210 mg of phenol per liter of urine, we may conclude that in the work room of this group the maximum allowable concentration of 25 ppm of benzene was not exceeded.

If the group under consideration is large, it will be possible to arrive at a reliable conclusion with regard to the benzene concentration in the atmosphere even if the method used to determine the phenol content is not

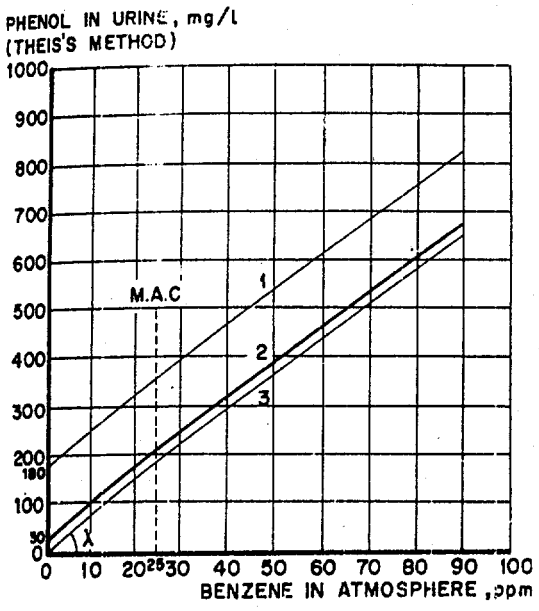

FIGURE 5. Relation between phenol secretion in urine and benzene concentrations in the atmosphere (Walkley *et al.*).

very exact. The routine examination by the industrial physician, however, will often include groups of workers from small departments, which consist of only a few persons. In that case there is the risk of erroneous conclusions being drawn on account of large variations in the $(p+m)$-cresol content of the urine.

If the slope of Walkley's correlation line is assumed to be correct, the limits of these variations in the Walkley graph can—on the basis of our data, which were obtained by the GLC method—be plotted as a "band" correlation (Figure 5, lines 1 and 3). The minimum content of phenol + cresol normally present in urine is 0 mg/liter, the maximum about 220 mg/liter (18+200, see Figures 1 and 2).

A cresol content of 200 mg/liter as found by the GLC method may show up as varying contents of apparent "phenol" in the colorimetric method of Theis and Benedict (depending on whether this cresol is *p*-cresol, *m*-cresol, or a mixture of the two, and also on the wavelength used). The maximum apparent "phenol" content resulting from 200 mg of cresol per liter that may be thus found is approximately 160 mg/liter. This adds up to the real phenol content; hence, we might roughly take an apparent "phenol" content of 180 mg/liter as the maximum value for urines of persons not exposed to benzene. Therefore, the upper limit of the band in Figure 5 has been drawn through a point on the ordinate corresponding to 180 mg of "phenol" per liter.

On comparing Figure 5 with Walkley's original figure, it will be seen that most of the scattered points in the latter plot fall well within the band formed by lines 1 and 3 of Figure 5. Walkley and her co-workers have indeed found that by Theis and Benedict's method "phenol" contents appreciably higher than 50 mg/liter may occasionally be determined in urines of persons not exposed to benzene; mention was made of some cases where the level was as high as 100 mg/liter or even 200 mg/liter in the absence of benzene exposure.

From Figure 5 it is seen that a urine "phenol" content up to 180 mg/liter, as determined by Theis and Benedict's method, does not necessarily point to benzene exposure. A benzene level between 180 and 360 mg/liter is found.

If a similar graph is drawn for the specific phenol content as determined by the GLC method—without the necessity of making allowance for the cresol content of urine—the

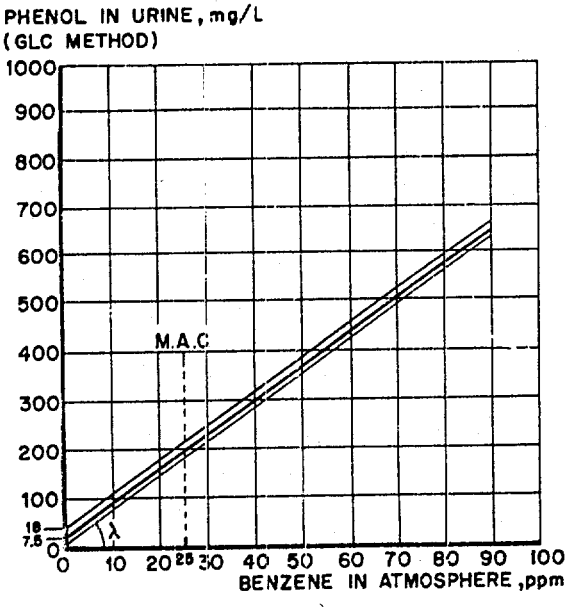

FIGURE 6. Relation between phenol secretion in urine and benzene concentrations in the atmosphere.

correlation lines shown in Figure 6 are found.

Again, the slope of the lines as given by Walkley (angle λ) is taken to be correct. The band is narrow, which allows of far more accurate detection of possible exposure to benzene. A phenol content of more than 10 mg/liter as a group average (even in small groups) is an indication of benzene exposure, and phenol values of more than 200 mg/liter indicate exposure to 25 ppm or more of benzene.

Although in composing the above graphs we took Walkley's correlation line (slope of the line) to be correct, there must, in our opinion, be some doubt as to its correctness on account of the low degree of accuracy of the Theis and Benedict method which Walkley used.

Our investigations clearly indicate that the GLC method, owing to its specificity and rapidity, is to be preferred to Theis and Benedict's method. It is also superior, though not so strongly, to Gibb's method, which, moreover, is time-consuming.

We think, therefore, that it would be desirable to repeat Walkley's investigation by using an exposure chamber and the GLC method.

Conclusion

At present the gas chromatographic method (GLC) is the most specific and, after preparation of the apparatus, also the most rapid method for determining the phenol content of urine. Gibb's method is not much inferior to GLC analysis, at least with regard to interference by *p*-cresol, but it is far more time-consuming. Theis and Benedict's method would be preferable to Gibb's if it were specific for phenol and if *p*-cresol were not co-determined.

If the correlation line (and the angle of its slope) as suggested by Walkley is correct, the results obtained by GLC also indicate that the limit of 200 mg of phenol per liter of urine may be considered medically safe.

It is advisable, however, to check the correctness of the correlation line with the aid of the GLC method to determine the phenol content of urine.

Addendum

After this article had been submitted, an as yet unpublished article by H. Buchwald (The Occupational Hygiene Service, Slough, England) came to the authors' notice. In this article it is pointed out that the average specific gravity of the urine of ethnic groups varies according to their geographical situation.

The correction for a specific gravity of 1.024, made by the authors in accordance with Elkins' advice and with the practice of many authors in various countries, is based on an extensive investigation made in the USA (Levine and Faby, 1945). According to recent research in England (Slough) and Ireland, the average specific gravity of urine in the British Isles is not 1.024 but 1.016.

This finding prompted the authors to determine the average specific gravity of the urine samples investigated. Although the number of samples was far too small to enable conclusions to be drawn from the results, the average specific gravity did come out to be 1.016, in agreement with British values. This means that the average normal urinary phenol content corrected for specific gravity, was not 7-8 but 5-6 mg/liter of urine for the persons investigated, which is practically equal to the uncorrected average.

References

1. Teisinger, J., and V. Fiserova-Bergerova: Valeur comparé de la determination des sulfates et du phenol contenus dans l'urine pour l'évaluation de la concentration du benzene dans l'air. *Arch. Maladies Profess. Med. Travail Sécurité Sociale 16:* 221 (1958).
2. Walkley, J. E., L. D. Pagnotto, and H. B. Elkins: The measurement of phenol in urine as an index of benzene exposure. *Amer. Ind. Hyg. Assoc. J. 22:* 362 (1961).
3. Gibbs, H. D.: Phenol Tests: III Endophenol Test. *J. Biol. Chem. 72:* 649 (1927).
4. Theis, R. C., and S. R. Benedict: Determination of Phenols in the Blood. *J. Biol. Chem. 61:* 67 (1924).
5. Williams, R. T.: *Detoxication Mechanisms.* 2nd Ed.,
6. See reference, 5, p. 293.
7. See reference 5, p. 294.
8. Portheous, J. W., and R. T. Williams: Studies in Detoxication. XIX: Metabolism of Benzene. *Biochem. J. 44:* 46 (1949).
9. Deichmann, W., and L. J. Schafer: Phenol Studies. *Amer. J. Clin. Path. 12:* 129 (1942).